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Published in:
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Chapter 6
The Role of Polycomb Group Proteins in Hematopoietic Stem Cell (HSC) Self-Renewal and Leukemogenesis

Vincent van den Boom, Hein Schepers, Annet Z. Brouwers-Vos, and Jan Jacob Schuringa

Abstract Throughout embryonic development as well as during adult hematopoiesis Polycomb group (PcG) proteins fulfill important functions. Stem cell self-renewal but also lineage fate decisions are controlled by PcGs. Besides a role in normal hematopoiesis, PcGs are often deregulated in various types of cancer, including human leukemias. Within this chapter we will discuss the current understanding of complex composition of canonical and noncanonical Polycomb repressive complexes, how these can contribute to normal hematopoiesis, and how PcG proteins can participate in leukemic transformation.

Keywords Polycomb repressive complex • Human hematopoietic stem cells • Leukemia • Noncanonical • Stem cell self-renewal • Differentiation • AML • ALL

6.1 Introduction

Polycomb group (PcG) proteins are involved in epigenetic repression of gene transcription and generally reside in two distinct complexes: Polycomb repressive complex 1 (PRC1) and 2 (PRC2) (Fig. 6.1) (Simon and Kingston 2009). According to the classical model for PcG-mediated repression, the PRC2 complex, containing the methyltransferase EZH2, EED, and SUZ12, first trimethylates histone H3 at lysine 27 (H3K27me3) (Cao et al. 2002; Kirmizis et al. 2004; Kuzmichev et al. 2002). This epigenetic modification recruits the five-subunit PRC1 complex, most likely via the chromobox domain of the CBX subunit of the PRC1 complex (Bernstein et al. 2006; Levine et al. 2002). Subsequently, the PRC1 complex, via its RING1 subunit, can ubiquitinate histone H2A at Lysine 119 (H2AK119ub)

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C. Bonifer and P.N. Cockerill (eds.), *Transcriptional and Epigenetic Mechanisms Regulating Normal and Aberrant Blood Cell Development*, Epigenetics and Human Health, DOI 10.1007/978-3-642-45198-0_6,

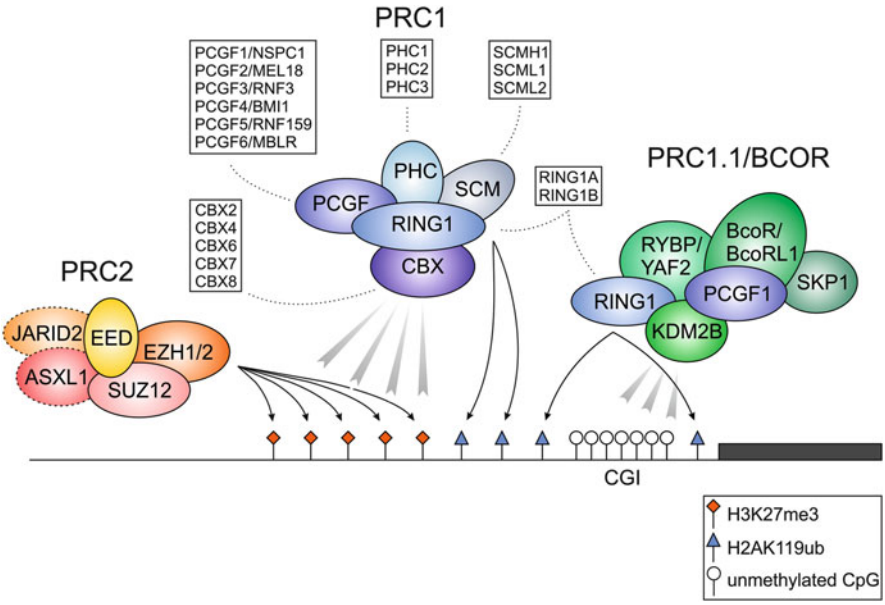

Fig. 6.1 Overview of canonical and noncanonical PcG complexes

(de Napoles et al. 2004; Wang et al. 2004). In humans, each of the PRC1 components has multiple paralog family members: six PCGF members (PCGF1/NSPC1, PCGF2/MEL18, PCGF3, PCGF4/BMI1, PCGF5, and PCGF6/MBLR), three PHC members (PHC1, PHC2, and PHC3), five CBX members (CBX2, CBX4, CBX6, CBX7, and CBX8), three Sex combs on midleg (SCM) members (SCML1, SCML2, and SMLH1), and two RING1 members (RING1A and RING1B). These paralogs allow a large diversity of distinct PRC1 complexes involved in PcG-mediated silencing (Whitcomb et al. 2007). This idea was supported by the identification of BMI1- and MEL18-containing PRC1 complexes and similarly PRC1 complexes with mutually exclusivity of CBX paralogs (Elderkin et al. 2007; Maertens et al. 2009; Vandamme et al. 2011). Indeed, PRC1 complexes were identified, which lack a CBX paralog family member but do contain RYBP or YAF2 and are targeted to PcG target genes independently of H3K27me3 (Gao et al. 2012; Tavares et al. 2012). Furthermore, some paralog family members like RING1A/B, PCGF1, and PCGF6 also reside in noncanonical PRC1 complexes such as the BCOR and E2F6 complexes (Gearhart et al. 2006; Ogawa et al. 2002; Sanchez et al. 2007; Trimarchi et al. 2001; Gao et al. 2012). Recently, the PRC1.1/BCOR complex was shown to target PcG genes by means of the KDM2B H3K36 demethylase (FBXL10), which specifically binds to unmethylated CpG islands (Fig. 6.1) (Farcas et al. 2012; He et al. 2013; Wu et al. 2013). These data show that noncanonical PRC1 complexes are involved in maintaining H2AK119ub levels as well. Also components of the core PRC2 complex display substoichiometric interactions with other (PcG) proteins. One such example is ASXL1, which is thought to mediate

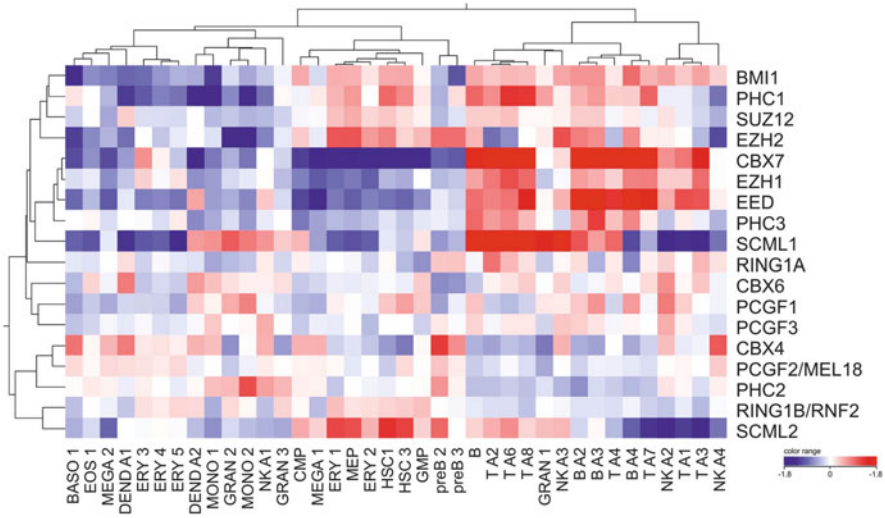

Fig. 6.2 Expression of PcG proteins in human CB cells throughout the hematopoietic compartment [based on data published by (Novershtern et al. 2011)]

recruitment and/or stabilization of the PRC2 complex to specific loci in the genome (Abdel-Wahab and Dey 2013).

The expression of PcG proteins throughout the hematopoietic compartment appears to be quite specific, with some PcGs being predominantly expressed in the immature stem/progenitor compartments, while other PcGs display a much more lineage-specific expression profile (Fig. 6.2). Taken together, these data suggest that many distinct PcG complexes exist that are likely to fulfill different functions. Yet, very little is known about possible differences in complex composition in, for instance, self-renewing hematopoietic stem cells (HSCs) versus non-self-renewing progenitors, or in normal versus leukemic stem cells (LSCs). Moreover, it is currently unclear whether specific loci would be preferentially occupied by certain PcG complexes across these different cell types. Lastly, we do not know exactly how repression of these different loci would be controlled by the various PRC complexes. Accumulating recent data indicates that molecular pathways regulating cell cycle, apoptosis, senescence, reactive oxygen species (ROS) metabolism and DNA repair are at least in part under control of PcG proteins. Whether PcG proteins fulfill similar roles in leukemia is currently unclear.

Understanding the molecular mechanisms by which PcG proteins affect stem cell fate will increase our insights into the biology of HSCs and will also aid in understanding the process of leukemic transformation, and ultimately in the identification of novel drug targets that might facilitate the eradication of LSCs. Here, we will provide an overview of the current understanding of the role of PcG proteins in HSC self-renewal and leukemogenesis.

6.2 Polycomb Function in Normal Hematopoiesis

6.2.1 Polycomb Repressive Complex 2

6.2.1.1 EZH1 and EZH2

Of all PRC2 complex subunits, the methyltransferase EZH2 has been studied in most detail. To study EZH2 function in murine hematopoiesis, conditional knockout models were required due to embryonic lethality of *Ezh2* knockout mice (O'Carroll et al. 2001). Using *Mx-Cre;Ezh2*$^{fl/fl}$ mice it was first shown that B cell development was impaired in an *Ezh2* null background (Su et al. 2003). More specifically, pre-B cell generation was affected and accumulation of immature B cells in the bone marrow (BM) was observed. This was caused by impaired rearrangement of the immunoglobin heavy chain gene leading to reduced μ-chain expression. Transplantation experiments emphasized that this phenotype was cell autonomous and niche independent. In contrast, pro-B cell development was unaffected indicating that EZH2 has stage-specific functions in B-lymphopoiesis. EZH2 also plays a role in T-lymphocyte generation as *Ezh2* null thymocytes fail to develop further than early CD4/CD8 double negative CD44^{int}/CD25^{hi} stage in the thymus in a transplant setting (Su et al. 2005). In contrast to adult BM cells, fetal liver cells are highly sensitive to deletion of *Ezh2* (Mochizuki-Kashio et al. 2011). Using a *Tie2-Cre;Ezh2*$^{fl/fl}$ conditional model it was shown that levels of LSK, CMP, GMP, and MEP cells were reduced in conditional *Ezh2*$^{-/-}$ whole fetal livers at E12.5, indicating an important role for EZH2 during fetal hematopoiesis. Strikingly, *Ezh2* null cells could efficiently reconstitute hematopoiesis in adult BM in a competitive transplant setting. In addition, H3K27me3 levels, which are very low in fetal liver *Ezh2* KO cells, were largely restored in *Ezh2*$^{-/-}$ fetal liver cells that reconstituted the BM of recipient mice. The authors suggest a model where *Ezh1*, which is strongly induced in adult versus fetal LSK cells, may rescue *Ezh2* null cells in an adult BM environment by inducing and/or maintaining proper H3K27me3 levels in the context of an EZH1–PRC2 complex (Mochizuki-Kashio et al. 2011). Murine EZH2 overexpression studies showed enhanced long-term repopulation potential in a serial transplant setting (Kamminga et al. 2006).

It was previously shown that in *Ezh2* null embryonic stem (ES) cells H3K27me3 levels are reduced but not completely lost (Shen et al. 2008). Furthermore, EZH1 was shown to interact with PRC2 components and locates to EZH2 target genes whereby most likely (lower) levels of the H3K27me3 mark can be maintained at these loci (Margueron et al. 2008; Shen et al. 2008). Conditional deletion of both *Ezh1* and *Ezh2* in the mouse skin showed a complete loss of H3K27me3 levels further supporting a model where *Ezh1* can at least partially compensate for loss of *Ezh2* (Ezhkova et al. 2011). Recently, the specific role of *Ezh1* in hematopoiesis was addressed in a hematopoietic conditional knockout model (Hidalgo et al. 2012). Conditional deletion of *Ezh1* in the hematopoietic system using a *Vav-Cre;Ezh1*$^{fl/fl}$ mouse model showed development of BM hypoplasia most likely

due to reduced numbers of LT-HSC, ST-HSC, MPP, and CLP cells, whereas CMP, GMP, and MEP cells were unaffected. Competitive transplantation experiments using a tamoxifen-inducible *Ezh1* conditional knockout model showed a loss of repopulating ability, which was attributed to increased levels of senescence in the primitive stem cell compartment. Increased senescence in this model is likely driven by de-repression of $p16^{INK4A}$ since a *Vav-Cre;Ezh1*$^{fl/fl}$*;Ink4a-Arf*$^{fl/fl}$ showed restoration of the senescence phenotype. Similar to *Ezh2* knockout mice, B-cell lymphopoiesis was also affected in *Ezh1* conditional knockout mice (Su et al. 2003; Hidalgo et al. 2012). In contrast to the *Ezh2* conditional knockout model, deletion of *Ezh1* in the hematopoietic system also led to a reduction in pro-B cell numbers. Furthermore, fetal liver hematopoiesis was not affected by *Ezh1* deletion, suggesting that *Ezh2* is indeed the dominant H3K27 methyltransferase during fetal embryogenesis (Mochizuki-Kashio et al. 2011; Hidalgo et al. 2012).

Taken together, EZH1 and EZH2 seem to have both separate as well as overlapping functions in the hematopoietic system, whereby the loss of one methyltransferase can be partially compensated for by the other depending on the hematopoietic cell lineage and developmental stage.

6.2.1.2 EED

Apart from the methyltransferases EZH1 and EZH2, the PRC2 complex is also composed of the core subunits EED and SUZ12. The role of EED in murine hematopoiesis was first described by Lessard and colleagues using mice heterozygous for an *Eed* null allele or homozygous for an *Eed* hypomorphic allele (Lessard et al. 1999). Previously, homozygous *Eed* null embryos were shown to exert severe gastrulation defects (Schumacher et al. 1996; Faust et al. 1995). Biochemical analysis showed that EED interacts with H3K27me3 via its carboxy-terminal domain and that this interaction stimulates PRC2 methyltransferase activity suggesting a role for EED in propagation of H3K27me3 through DNA replication (Margueron et al. 2009). In murine hematopoiesis, EED likely acts as a negative regulator of cell proliferation and *Eed*$^{3354/+}$ mice showed increased levels of myeloid progenitors and pre-B cells. In addition, at older age these mice displayed hyperproliferation of both lymphoid and myeloid cells (Lessard et al. 1999). However, classical target genes like $p16^{INK4A}$ and $p19^{ARF}$ and Hox genes were not upregulated in this mouse model (Lessard et al. 1999).

6.2.1.3 SUZ12

Similar to *Ezh2* null and *Eed* null mice, also homozygous deletion of the *Suz12* gene resulted in early embryonic lethality (Pasini et al. 2004). This was accompanied by a complete loss of H3K27me2/3 suggesting that PRC2 activity is completely lost in these embryos. In murine hematopoiesis, an ENU-induced loss-of-function mutation in the *Suz12* gene was identified as a suppressor of thrombocytopenia and HSC

defects in *cMpl*$^{-/-}$ mice (Majewski et al. 2008). More recently, it was shown that crossing heterozygous *Eed* null or *Ezh2* null mutations in a *cMpl*$^{-/-}$ background also resulted in an improvement in the thrombocytopenic phenotype, whereby increased white blood cell counts were observed (Majewski et al. 2010).

6.2.2 Polycomb Repressive Complex 1

As described above, the PRC1 complex consists of five subunits (PCGF, CBX, PHC, RING1, and SCM), and each of these subunits has a family of paralogs both in mice and humans. Below we will describe the involvement of the various paralog family members in hematopoiesis.

6.2.2.1 PCGF Paralog Family

The PCGF paralog family is composed of PCGF1 (NSPC1), PCGF2 (MEL18), PCGF3, PCGF4 (BMI1), PCGF5, and PCGF6 (MBLR). Of these, BMI1 has been studied most extensively in hematopoiesis. BMI1 was initially observed to be oncogenic in a retroviral integration site screen where it was identified as a collaborating hit in MMLV-induced B-cell lymphomas in E*μ-myc* transgenic mice (Haupt et al. 1991; van Lohuizen et al. 1991). Homozygous deletion of *Bmi1* in mice resulted in reduced numbers of hematopoietic progenitors and more differentiated cells, eventually leading to hematopoietic failure (van der Lugt et al. 1994). More detailed analysis showed that BMI1 has a central regulatory role in self-renewal of HSCs by inducing symmetrical cell division both in mouse and human model systems (Park et al. 2003; Iwama et al. 2004; Lessard and Sauvageau 2003; Rizo et al. 2008). Accordingly, *Bmi1*$^{-/-}$ mice displayed dramatically reduced HSC frequencies. Mechanistically, the role of BMI1 in regulating HSC self-renewal is partially explained by its ability to repress the *Ink4a-Arf* locus (Jacobs et al. 1999; Park et al. 2003). Expression of p16^{INK4A} and p19^{ARF} in HSCs induces cell cycle arrest and p53-mediated cell death. Loss of BMI1 expression most likely results in a decreased H2AK119 ubiquitinating activity of the PRC1 complex at the *Ink4a-Arf* locus, inducing expression of p16^{INK4A} and p19^{ARF}. Importantly, the hematopoietic phenotype of *Bmi1*$^{-/-}$ mice is not only dependent on the induction of Ink4a/Arf. *Bmi1*$^{-/-}$;*Ink4a-Arf*$^{-/-}$ double knockout mice showed a partial recovery of hematopoietic cell counts but did not show a complete reversal of the *Bmi1* null phenotype, suggesting that other pathways are also involved (Bruggeman et al. 2005). In a separate study, competitive transplant experiments showed that *Bmi1*$^{-/-}$;*Ink4a-Arf*$^{-/-}$ cells showed peripheral blood chimerism levels comparable to wild-type cells, whereas *Bmi1*$^{-/-}$ cells did not contribute at all (Oguro et al. 2006). Although HSC self-renewal clearly correlated with p16^{INK4A} and p19^{ARF} expression, the typical hypoplastic BM phenotype of *Bmi1* null mice was not completely rescued by deletion of the *Ink4a-Arf* locus.

Gene expression analysis of the HSC/MPP fraction in *Bmi1* null mice surprisingly showed premature transcriptional activation of Ebf1 and Pax5, two regulators of B cell lymphopoiesis (Oguro et al. 2010). BMI1 is directly recruited to the promoters of these genes and *Bmi1*$^{-/-}$;*Ink4a-Arf*$^{-/-}$ cells are biased toward the B cell lineage at the expense of T cell lymphopoiesis.

A candidate *Ink4a-Arf*-independent pathway for BMI1 in controlling HSCs is regulation of ROS in the cell. *Bmi1*$^{-/-}$ mice displayed impaired mitochondrial function due to increased expression of PcG target genes involved in ROS metabolism (Liu et al. 2009). Long-term HSCs from *Bmi1*$^{-/-}$ mice showed increased levels of ROS and treatment of mice with the antioxidant *N*-acetylcysteine (NAC) resulted in a rescue of thymocyte cell numbers compared to non-treated *Bmi1*$^{-/-}$ mice. Furthermore, de-regulated ROS metabolism induced activation of the DNA damage response pathway. Knockout of *Chk2*, a component of the DNA damage response pathway, in a *Bmi1*$^{-/-}$ background resulted in partial reversal of the thymocyte phenotype observed in *Bmi1*$^{-/-}$ mice and increased LSK cell numbers in the BM. However, *Bmi1*$^{-/-}$;*Chk2*$^{-/-}$ cells failed to give long-term repopulation in a competitive transplant setting similar to *Bmi1*$^{-/-}$ mice, indicating that the HSC self-renewal phenotype of *Bmi1* null mice is not rescued by *Chk2* deletion. Interestingly, knocking down BMI1 in CD34^{+} human cord blood cells also induced increased ROS levels and apoptosis (Rizo et al. 2009). These data clearly show that apart from the role of BMI1 in regulating cell cycle and senescence through the *Ink4a-Arf* locus, BMI1 is also implicated in other pathways regulating oxygen metabolism.

PCGF1 has previously been identified as a member of the BCOR complex, containing RING1A, RING1B, BCOR, SKP1, and KDM2B (FBXL10) (Gearhart et al. 2006). Recently, the H3K36-specific demethylase KDM2B was shown to target this noncanonical PRC1 complex to unmethylated CpG islands in the promoters of lineage-specific genes in ES cells (Fig. 6.1) (Farcas et al. 2012; He et al. 2013; Wu et al. 2013). Depletion of KDM2B resulted in derepression, a loss of RING1B binding and decreased H2AK119ub levels at these target genes. In mouse hematopoietic cells, PCGF1 was picked up as a factor negative regulating self-renewal of lineage negative cells in a *Runx1* conditional knockout setting (Ross et al. 2012). PCGF1 knockdown was shown to induce expression of HOXA cluster genes and led to a loss of H2AK119ub at the promoters of these genes. Knockdown of PCGF1 in human CD34^{+} CB cells was recently shown to give a mild growth reduction in in vitro cultures and a loss of CFC frequency (van den Boom et al. 2013).

Mel18$^{-/-}$ mice display severe posterior transformations of the axial skeleton in a manner similar to *Bmi1* null mice (Akasaka et al. 1996; van der Lugt et al. 1994). Similarly, analysis of the hematopoietic compartments showed a strong reduction in cellularity in the thymus and spleens in both knockout models. Both models do not completely overlap in terms of phenotype since *Mel18* null mice develop abnormalities of the lower intestine, whereas *Bmi1*-deficient mice specifically display a cerebellum defect. However, generation of *Mel18*$^{-/-}$;*Bmi1*$^{-/-}$ mice

showed that both proteins synergistically regulate *Hox* cluster expression (Akasaka et al. 2001). Competitive transplants using E14 fetal liver cells from *Mel18*$^{-/-}$ mice showed a only a mild reduction in repopulating activity compared to a severe loss of repopulating activity in *Bmi1*$^{-/-}$ cells (Iwama et al. 2004). Knockdown of MEL18 in human cord blood (CB) cells resulted in a dramatic reduction in proliferation in both BM stromal cocultures and liquid cultures and a strong reduction in CFC plating efficiency (van den Boom et al. 2013). However, while BMI1 depletion led to an upregulation of p14^{ARF} and p16^{INK4A}, MEL18 knockdown did not induce the expression of these genes. These data show that, although both involved in the regulation of hematopoiesis, BMI1 and MEL18 have distinct functions and BMI1 clearly has a much more prominent role in maintaining HSC self-renewal.

Concerning PCGF3, PCGF5, and PCGF6 no information is currently available on their potential function in murine hematopoiesis. PCGF6 knockdown in human CD34^{+} CB cells did not or only mildly affect cell growth in hematopoietic growth assays and did not alter CFC plating efficiency, suggesting that this gene is not involved in human hematopoiesis (van den Boom et al. 2013).

6.2.2.2 CBX Paralog Family

The CBX paralog family encompasses the CBX2, CBX4, CBX6, CBX7, and CBX8 proteins, which are homologues of the *Drosophila melanogaster* Polycomb protein and target the PRC1 complex to the chromatin through their interaction with H3K27me3. However, the chromodomains of the various CBX proteins show significant differences in substrate specificity in in vitro assays, since some interact with both H3K27me3 and H3K9me3, or selectively with H3K9me3 (Bernstein et al. 2006). Recently, two independent studies reported different functional modalities between CBX paralog family members in mouse ES cells (Morey et al. 2012; O'Loghlen et al. 2012). These data showed a key role for CBX7 in undifferentiated ES cells, whereas CBX2 and CBX4 were essential for lineage commitment. Gene expression data from human hematopoietic cell subsets also showed that the various CBX proteins are differentially expressed in HSCs, progenitors, and differentiated cells (Fig. 6.2) (van den Boom et al. 2013; Novershtern et al. 2011).

Two *Cbx2* (*M33*) knockout models were generated both of which showed skeletal transformations (Katoh-Fukui et al. 1998; Core et al. 1997). Interestingly, Coré and colleagues observed a reduction of cell numbers in the spleen, thymus, and BM of *Cbx2*$^{-/-}$ mice and found both B and T cell lymphopoiesis to be affected (Core et al. 2004; Core et al. 1997). However, competitive transplant experiments using fetal liver cells from *Cbx2*$^{-/-}$ mice did not show any change in repopulation capability compared to wild-type cells (Iwama et al. 2004). In contrast, knockdown of CBX2 in human CD34^{+} CB cells resulted in a severe phenotype in both the HSC and progenitor compartment (van den Boom et al. 2013). CBX2 knockdown cells displayed a reduced proliferation and increased apoptosis. Furthermore, the *CDKN1A* gene (*p21*) was directly targeted by CBX2 and concurrent knockdown of CBX2 and p21 partially rescued the CBX2 knockdown phenotype. It is possible

that these differences in CBX2 function between human and mouse hematopoiesis are a consequence of species-specific differences in CBX2 function. For example, where p21 is a bona fide target of CBX2/PRC1 and PRC2 complexes in human $CD34^+$ CB cells, it has not been annotated as a PcG target gene in mouse cells.

CBX4 has the unique biochemical property among other CBXs that it is a SUMO E3 ligase stimulating UBC9-dependent sumoylation of the transcriptional repressor CtBP by tethering both to Polycomb bodies (Kagey et al. 2003). Interestingly, in human epidermal stem cells CBX4 preserved a slow-cycling and undifferentiated state and also prevents from senescence induction (Luis et al. 2011). Recent generation of a conditional *Cbx4* knockout mouse model showed that thymic development was impaired. However, this phenotype was caused by dysfunction of the thymic epithelial cells rather than a consequence of intrinsic defects of the developing thymocytes (Liu et al. 2013). CBX4 overexpression in murine hematopoietic cells mildly suppressed proliferation, inhibited replating potential in CFC assays, and reduced CAFC activity (Klauke et al. 2013). In contrast, knockdown of CBX4 in human $CD34^+$ CB cells strongly reduced cell proliferation in BM stromal cocultures and liquid cultures and diminished CFC plating efficiency (van den Boom et al. 2013).

Cbx7 knockout mice are born in Mendelian ratios and display a slight increase in body length (Forzati et al. 2012). MEFs from these mice showed increased proliferation and decreased induction of senescence and adult $Cbx7^{-/-}$ mice develop tumors in the liver and the lungs. However, effects of *Cbx7* knockout on the hematopoietic system were not studied in this report. Contrasting the tumor-suppressor role of CBX7 in other tissues, overexpression of CBX7 in murine hematopoietic cells increases proliferation in liquid cultures and enhances the in vitro proliferative capacity of LT-HSCs and ST-HSCs (Klauke et al. 2013). CBX7 overexpression in LT-HSCs and subsequent transplantation showed enhanced numbers of ST-HSCs and MPPs but not LT-HSCs at late stages after transplantation. The authors suggest a model where CBX7 preserves a HSC self-renewing state by specifically repressing genes involved in differentiation. CBX7 knockdown in human CB $CD34^+$ cells led to a mild proliferative disadvantage and reduction in CFC frequencies (van den Boom et al. 2013).

To study the role of CBX8 in normal hematopoiesis, both constitutive and conditional CBX8 knockout models were used (Tan et al. 2011). CBX8 deletion did not lead to changed peripheral blood cells numbers and bone marrow cellularity. Furthermore, LT-HSC functionality was not affected as shown by competitive transplant assays, suggesting that CBX8 is not involved in normal hematopoiesis. In contrast, in a separate study, overexpression of CBX8 in murine hematopoietic cells showed a phenotype opposite of CBX7, where frequencies of LT-HSCs, ST-HSCs, and MPP are all decreased (Klauke et al. 2013). Knockdown of CBX8 in human $CD34^+$ CB cells resulted in a mild negative phenotype in terms of cell proliferation, similar to CBX7 knockdown (van den Boom et al. 2013). Myeloid differentiation was not affected by knockdown of either CBX7 or CBX8.

6.2.2.3 RING1 Paralog Family

The RING1 paralog family consists of RING1A and RING1B. Both proteins possess H2AK119 ubiquitinating activity (Buchwald et al. 2006; de Napoles et al. 2004; Wang et al. 2004). Deletion of both *Ring1a* and *Ring1b* was necessary to achieve genome-wide depletion of H2AK119ub (Endoh et al. 2008; Stock et al. 2007). Furthermore, H2A ubiquitination is essential for repression of developmental genes and preserving ES cell identity (Endoh et al. 2012). Although H2AK119 ubiquitination is in part BMI1 dependent, recent studies showed RING1B-dependent H2AK119 ubiquitination in the context of noncanonical PRC1 complexes (*i.e.,* PRC1-RYBP, BCOR complexes) that are targeted to the chromatin independently of H3K27me3 (Farcas et al. 2012; He et al. 2013; Wu et al. 2013; Kallin et al. 2009). *Ring1a*$^{-/-}$ mice showed skeletal abnormalities and slight deregulation of Hox gene expression (del Mar Lorente et al. 2000). In contrast, where *Ring1a* null mice are viable, deletion of *Ring1b* led to a severe gastrulation defects and embryonic lethality (Voncken et al. 2003). The generation of a conditional *Mx-Cre;Ring1b*$^{fl/fl}$ mouse model allowed the investigation of Ring1B function in hematopoiesis (Cales et al. 2008). This study showed that *Ring1b* depletion led to a mild increase in the primitive stem/progenitor compartment (LSK, Lin$^-$ cells), whereas the total BM compartment was slightly decreased. In line with this phenotype, p16^{INK4A} was selectively activated in more differentiated cells but not in the Lin$^-$ compartment, whereas the positive cell cycle regulator CycD2 was upregulated in most compartments. Simultaneous deletion of *p16*^{INK4A}, *p19*^{ARF}, and *Ring1b* resulted in a rescue of the negative effects on proliferation of the mature compartments, although the hyperproliferative phenotype of the primitive compartment was not corrected. Knockdown of RING1B in human CB CD34$^+$ cells showed severe defects in long-term expansion and progenitor frequencies (van den Boom et al. 2013).

6.2.2.4 PHC Paralog Family

Both in mice and humans the Polyhomeotic paralog family encompasses three members: PHC1, PHC2, and PHC3. *Phc1*$^{-/-}$ (*Rae28*) mice showed skeletal abnormalities and a reduced spleen size (Takihara et al. 1997). Further studies showed involvement of *Phc1* in early B cell lymphopoiesis (Tokimasa et al. 2001). Furthermore, *Phc1* null mice displayed reduced hematopoietic progenitor activity in the fetal liver and loss of long-term repopulating activity of fetal liver cells in competitive transplant experiments (Ohta et al. 2002; Kim et al. 2004). *Phc2*$^{-/-}$ mice also showed skeletal abnormalities and deregulated expression of Hox genes and p16^{INK4A} and p19^{ARF} (Isono et al. 2005). In contrast to *Phc1*, null mice deletion of *Phc2* did not have an apparent phenotype in hematopoiesis.

6.2.2.5 SCM Paralog Family

The sex combs on midleg (SCM) paralog family consists of SCMH1, SCML1 and SCML2. SCMH1 was first identified as a substoichiometric subunit of the PRC1 complex (Levine et al. 2002). Protein interaction studies have showed that also SCML1 and SCML2 of this paralog family can interact with other PRC1 subunits in human cells (van den Boom et al. 2013; Gao et al. 2012). *Scmh1*$^{-/-}$ mice display characteristic features resembling other PcG knockout mouse models like skeletal abnormalities; however, studies concerning the hematopoietic system have not been reported (Takada et al. 2007).

6.3 Polycomb Function in Leukemia

6.3.1 Polycomb Repressive Complex 2

6.3.1.1 EZH1 and EZH2

Unlike its related family member EZH1, a potential role for the PRC2 member EZH2 in hematological malignancies has been heavily investigated. Both tumor suppressor as well as oncogenic functions have been described, which will be discussed here. Besides being overexpressed in a variety of malignancies (Bachmann et al. 2006), EZH2 has shown aberrant expression in mantle cell lymphoma (Visser et al. 2001), Hodgkin lymphoma (Raaphorst et al. 2000), and non-Hodgkin lymphoma (Table 6.1) (van Kemenade et al. 2001). In complex karyotype AML, overexpression of EZH2 has also been observed (Grubach et al. 2008), although in a panel of 60 AMLs separated in $CD34^+$ and $CD34^-$ fractions, higher expression was only observed within the $CD34^-$ fraction, while the expression was significantly reduced within the $CD34^+$ compartment compared to normal bone marrow $CD34^+$ cells (Fig. 6.3) (Bonardi et al. 2013; de Jonge et al. 2011). In contrast, overexpression of EZH1 was observed within the AML $CD34^+$ compartment compared to NBM $CD34^+$ cells (Bonardi et al. 2013; de Jonge et al. 2011). More recently, mutations in the *EZH2* gene were discovered in ~22 % of follicular and diffuse large B cell lymphomas. Mutation of Tyrosine residue 641 (Y641) in the SET domain led to a severe decrease in enzymatic H3K27 trimethylation activity (Morin et al. 2010). EZH2 mutations were also reported for various myeloid malignancies like CMML, MDS, and AML (Abdel-Wahab et al. 2011; Ernst et al. 2012; Makishima et al. 2010; Cancer Genome Atlas Research Network. 2013). Although Y641 mutations were not found, these myeloid malignancies carried other *EZH2* mutations (R690, N693 and H694) that also affected the SET domain and thereby H3K27 trimethylation activity (Abdel-Wahab et al. 2011; Ernst et al. 2012; Makishima et al. 2010). In T-ALL ~18 % of the patients displayed truncating or missense mutations before the SET domain in

Table 6.1 Involvement of PcG proteins in human hematological malignancies

Canonical PRC complex	Protein	Disease	Aberrancy	ref
PRC2	EZH1	AML	Overexpressed (AML CD34$^+$)	(de Jonge et al. 2011)
	EZH2	AML	Overexpressed (AML CK)	(Grubach et al. 2008)
		AML	Reduced expression (AML CD34$^+$)	(de Jonge et al. 2011)
		AML	Reduced expression in CBF-mutated AML	(Grubach et al. 2008)
		AML, MDS, MPN, CMML	Loss-of-function mutations SET domain (R690, N693, H694)	(Abdel-Wahab et al. 2011;Ernst et al. 2012;Makishima et al. 2010;Ernst et al. 2010;Nikoloski et al. 2010)
		MCL, NHL, HL	Overexpressed	(Visser et al. 2001;Raaphorst et al. 2000;van Kemenade et al. 2001)
		FL,DLBCL	Loss-of-function mutations SET domain (Y641)	(Morin et al. 2010)
		T-ALL	Missense mutations	(Ntziachristos et al. 2012)
		T-ALL	Reduced expression	(Simon et al. 2012)
		ETP-ALL	Loss-of-function deletion/mutations SET domain (R684)	(Zhang et al. 2012)
	EED	MDS/MPN	Loss-of-function deletion/mutations (protein stability, EZH2 interaction, H3K27 me3 binding)	(Ueda et al. 2012)
		ETP-ALL	Loss-of-function deletion/mutations (R684)	(Zhang et al. 2012;Ueda et al. 2012)
	SUZ12	T-ALL	Loss-of-function deletion/mutations (S369fs, others)	(Ntziachristos et al. 2012;Zhang et al. 2012)
		MPN	Loss-of-function deletion/mutations	(Brecqueville et al. 2012;Score et al. 2012)
PRC2 interactors	ASXL1	MDS, MPN, AML, CML	Mutations (decrease protein stability)	(Abdel-Wahab et al. 2011;Shih et al. 2012;Abdel-Wahab et al. 2012;Schnittger et al. 2013)
	JARID2	MDS/MPN, AML	Deletion	(Puda et al. 2012)
PRC1	PCGF2/MEL18	AML	Overexpressed (AML CK)	(Grubach et al. 2008)
		AML	Reduced expression in CBF-mutated AML	(Grubach et al. 2008)
	PCGF4/BMI1	AML	Overexpressed (AML CD34$^+$)	(de Jonge et al. 2011;Chowdhury et al. 2007;van Gosliga et al. 2007)
		AML	Overexpressed, predicts prognosis	(Chowdhury et al. 2007)
		MDS	Overexpressed, predicts prognosis	(Mihara et al. 2006;Xu et al. 2011)
		CML	Overexpressed, predicts prognosis	(Mohty et al. 2007)
		HL, NHL	Overexpressed	(Raaphorst et al. 2000;van Kemenade et al. 2001;Dukers et al. 2004)
	CBX7	FL	Overexpressed	(Scott et al. 2007)
		AML	Reduced expression (AML CD34$^+$)	(de Jonge et al. 2011)
	RING1A	AML	Overexpressed (AML CD34$^+$)	(de Jonge et al. 2011)
		AML/MDS	Overexpressed	(Xu et al. 2011)
	PHC1/RAE28	B-ALL	Reduced expression	(Tokimasa et al. 2001)
		AML	Overexpressed (AML CD34$^+$)	(de Jonge et al. 2011)
Non-canonical PRC complex	**Protein**	**Disease**	**Aberrancy**	
PRC1.1/BCOR complex	BCOR	AML	Mutated	(Grossmann et al. 2011)
		AML	Overexpressed (AML CD34$^+$)	(Bonardi et al. 2013;de Jonge et al. 2011)
	BCORL1	AML	Mutated	(Li et al. 2011)
Role of PcGs proteins in hematological malignancy model systems				
gene	**model**		**phenotype**	
EZH2	Conditional Ezh2$^{-/-}$ mouse model		T-ALL	(Simon et al. 2012)
	mBM MLL-AF9 Tx model		Leukemia development in 2nd mice impaired in Ezh2$^{-/-}$ cells	(Neff et al. 2012;Tanaka et al. 2012)
	mBM Ezh2 overexpression		Myeloproliferative disease,	(Herrera-Merchan et al. 2012)

(continued)

Table 6.1 (continued)

EED	mBM MLL-AF9/NRAS(G12D) Tx model	Reduced leukemic growth upon EED knockdown	(Shi et al. 2012)
SUZ12	mBM MLL-AF9/NRAS(G12D) Tx model	Reduced leukemic growth upon SUZ12 knockdown	(Shi et al. 2012)
PCGF4/BMI1	Primary AML	Reduced long-term growth upon BM1 knockdown	(Rizo et al. 2009)
	huCB $CD34^+$ BCR-ABL/BMI1	Myeloid/lymphoid transformation in vitro, B-ALL in vivo	(Rizo et al. 2010)
	mBM BCR-ABL/Bmi1 Tx model	B-ALL	(Sengupta et al. 2012;Waldron et al. 2011)
	mBM MYC/Bmil1 Tx model	B-ALL	(van Lohuizen M. et al. 1991)
	mBM HoxA9/Meis1 Tx model	Loss of serial transplantation in $BMI1^{-/-}$ BM	(Lessard and Sauvageau 2003)
	mBM AML1-ETO Tx model	Loss of leukemic CFC replating in $BMI1^{-/-}$ BM	(Smith et al. 2011)
	mBM PLZF-RARα Tx model	Loss of leukemic CFC replating in $BMI1^{-/-}$ BM	(Smith et al. 2011)
	mBM MLL-AF9 Tx model	Loss of transformation in *HoxA9*$^{-/-}$/*Bmi1*$^{-/-}$ background	(Smith et al. 2003)
CBX8	mBM MLL-AF9/ENL Tx model	Loss of transformation in *Cbx8*$^{-/-}$ background	(Tan et al. 2011b)
ASXL1	mBM NRAS(G12D) Tx model	loss of ASXL1 accelerated onset of leukemia	(Abdel-Wahab et al. 2012)

AML acute myeloid leukemia, *MDS* myelodysplastic syndrome, *MPN* myeloproliferative neoplasms, *CMML* chronic myelomonocytic leukemia, *MCL* mantle cell lymphoma, *NHL* non-hodgkin lymphoma, *HL* hodgkin lymphoma, *DLCLB* diffuse large B-cell lymphoma, *T-ALL* T cell acute lymphoblastic leukemia, *ETP-ALL* Early T-cell precursor acute lymphoblastic leukemia, *FL* follicular lymphoma, *CML* chronic myeloid leukemia, *mBM* mouse bone marrow; *Tx* transplantation; *CB* human cord blood; *CK* complex karyotype

EZH2 (Ntziachristos et al. 2012). Furthermore, 7 % of the T-ALLs demonstrated alterations in another PRC2 member, SUZ12 (Ntziachristos et al. 2012). Although no deletions or mutations of *EZH2* could be found in the remaining human T-ALL cases, transcriptome analysis indicated that EZH2 expression levels were strongly reduced (Simon et al. 2012). Conditional deletion of *Ezh2* in a mouse model led to the occurrence of T-ALL leukemias (Simon et al. 2012). H3K27 di- and trimethylation were reduced, but not absent, indicating that EZH1 may partially compensate for the loss of EZH2 (Simon et al. 2012). These data suggest that EZH2 functions as a tumor suppressor in T-ALL and that loss of EZH2 contributes to this malignancy. In a large proportion of the T-ALLs with *EZH2* or other PRC2 mutations, also oncogenic mutations in *NOTCH1* were observed (65 %) (Ntziachristos et al. 2012). This suggests that *NOTCH1* mutations and *EZH2* mutations collaborate in the induction of T-ALL.

In contrast, in an MLL-AF9 model of leukemia, deletion of *Ezh2* did not affect leukemic initiation, but maintenance of the leukemia was impaired. This is most likely the result of a reduction in the frequency of LSCs (Neff et al. 2012; Tanaka et al. 2012). H3K27me3 levels were strongly reduced, but not absent in these cells (Neff et al. 2012; Tanaka et al. 2012). As leukemic growth was not completely absent and H3K27 trimethylation persisted on a subset of genes, also here EZH1 may partially compensate for the loss of EZH2 (Neff et al. 2012; Shi et al. 2013). These results imply that EZH2 is required for the proper maintenance of MLL-AF9-dependent LSCs, which is opposite from its tumor suppressor function in T-ALL. As also knockdown of Eed and Suz12 led to a reduction in MLL-AF9/NRASG12D-induced leukemic growth, this further strengthens the dependence of these

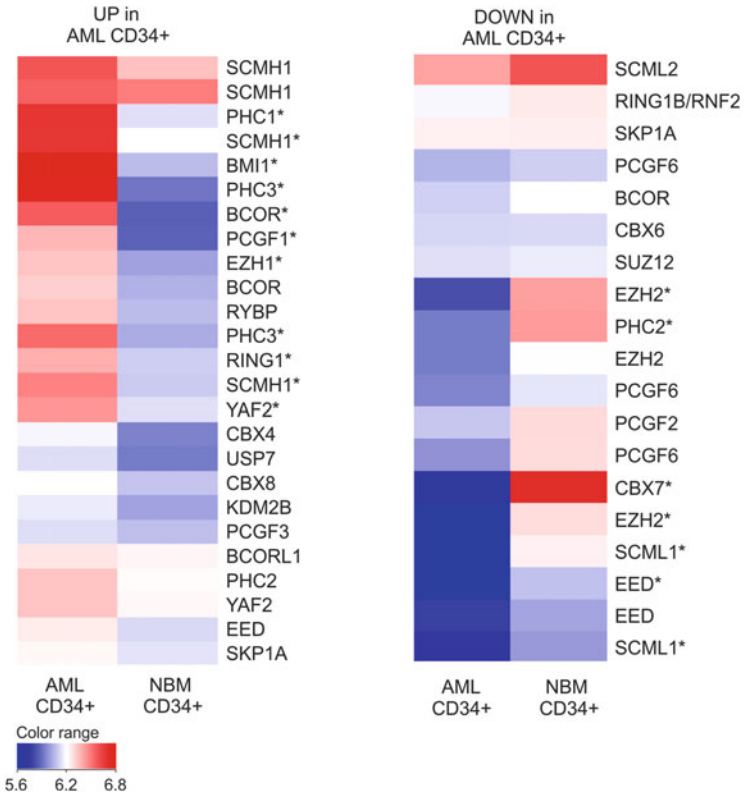

Fig. 6.3 Expression of PcG proteins in AML CD34$^+$ and NBM CD34$^+$ cells. Transcriptome profiling was performed on AML CD34$^+$ (n = 60) and normal BM CD34$^+$ cells ($n = 40$) (Bonardi et al. 2013; de Jonge et al. 2011). Differentially expressed genes were identified using an unpaired t-test with multiple testing correction (Benjamini-Hochberg, $p < 0.01$) and are marked with an *asterisk*

leukemias on PRC2 function (Shi et al. 2013). MLL-AF9-induced gene expression has been suggested to resemble an ES-like gene expression signature, which also comprises a PRC2 and MYC module (Kim et al. 2010; Somervaille et al. 2009). This MYC module is strongly reduced upon deletion of *Ezh2*, but only in secondary and not in primary leukemias. This suggests that PRC complexes play a role in the MLL-AF9-induced MYC module expression necessary for disease progression (Neff et al. 2012). In contrast, *EZH2*-deficient T-ALLs show an enhanced expression of MYC (Simon et al. 2012), suggesting that MLL-AF9 changes the gene regulatory functions of EZH2. Recent data showed that Menin, a partner of Mixed Lineage Leukemia (MLL), binds to the *Ezh2* promoter and enhances its expression (Thiel et al. 2013). Enhanced expression of EZH2 results in a myeloproliferative disease (Herrera-Merchan et al. 2012), but together with Menin causes a block in

myeloid differentiation (Thiel et al. 2013). Since mutations that affect EZH2 methyltransferase activity have also been shown to induce MPD/MDS (Abdel-Wahab et al. 2011; Makishima et al. 2010), this suggests that this activity needs to be carefully regulated, as both hypo- and hyperactivity can result in myeloid malignancies. Therefore, the contrasting role of EZH2 as a tumor suppressor or oncogene can most likely be explained by the different genetic context in which EZH2 plays a role. The fact that PRC2 complexes are recruited by PML-RARα and PLZF-RARα to RAREs while PRC1 is only recruited by PLZF-RARα further adds to this notion (Boukarabila et al. 2009; Villa et al. 2007).

6.3.1.2 EED

Unlike *Ezh2*, deletion of *Eed*, another PRC2 family member, completely abrogated MLL-AF9-mediated leukemia initiation (Neff et al. 2012). But although primary MLL-AF9 targets were not affected by *Ezh2* deletion, *Eed* deletion did affect the expression of these primary targets. These discrepancies can be reconciled with the following assumptions: 1; EZH1 partially can compensate for EZH2 activity and 2; both EZH1 and EZH2 activity is dependent upon EED. The first assumption has been confirmed in an MLL-AF9/NRASG12D leukemic model, where reduction in both EZH1 and EZH2 were necessary to reduce leukemic growth in vitro (Shi et al. 2013). Furthermore, EZH2 activity is dependent on EED (Denisenko et al. 1998) and EED mutants have been observed in patients with myelodysplastic syndrome and early T-cell precursor ALL (Ueda et al. 2012; Zhang et al. 2012). These mutations in EED will affect EZH1 and EZH2 activities and thereby contribute to aberrant myelopoiesis and lymphopoiesis. Recent evidence for this hypothesis stems from the fact that besides *EZH2* mutations in ALL, also *EED* and *SUZ12* mutations have been discovered in early T-cell precursor ALL (Zhang et al. 2012), indicating that it is the overall PRC2 activity that is inactivated. Although EED and SUZ12 knockdown reduced in vitro leukemic growth of MLL-AF9/NRASG12D cells, the reduction in in vivo leukemia development was rather modest (Shi et al. 2013). Together this suggests that PRC2 activity is required for leukemic expansion, rather than leukemic engraftment.

6.3.1.3 SUZ12

A role for SUZ12 in T-ALL development can be extrapolated from the fact that in ~7–12 % of the T-ALL cases, loss of function mutations was detected (Ntziachristos et al. 2012; Zhang et al. 2012). Downregulation of SUZ12 resulted in the expression of NOTCH1 target genes, comparable to EZH2 downregulation (Ntziachristos et al. 2012). The observation that ~33 % of early T-cell precursor ALLs has mutations in *SUZ12*, *EED*, and *EZH2* suggests that PRC2 has a tumor suppressor function in NOTCH1-mediated T-ALL.

In myeloid malignancies such as CML or other myeloproliferative neoplasms (MPNs), the role of SUZ12 is less clear. In non-CML MPNs, like polycythemia vera (PV), essential thrombocytopenia, (ET) and myelofibrosis (MF), rare mutations in *SUZ12* have been observed (Brecqueville et al. 2012; Score et al. 2012), but a higher incidence (31 %) of *ASXL1* mutations was identified (Brecqueville et al. 2012). As *ASXL1* mutations have been shown to result in loss of PRC2 activity (Abdel-Wahab et al. 2012), it is likely that these are mutually exclusive, since they all point to a tumor suppressive mechanism of PRC2 in these non-CML MPNs. This is consistent with deletion of another substoichiometric PRC2 member JARID2 in MPNs (Puda et al. 2012) and these data are in line with a tumor suppressive function of PRC2.

However, progression of chronic phase CML has been linked to the increased expression of SUZ12 in a WNT-dependent manner (Pizzatti et al. 2010). Knockdown of SUZ12 induced differentiation of these chronic phase blasts, indicating that PRC2 activity blocks differentiation. Similar, in an MLL-AF9/NRASG12D model of myeloid leukemia, reduction of SUZ12 severely hampered leukemic expansion, as did reduction in EED and EZH2 (Shi et al. 2013). These data are more in line with an oncogenic role of PRC2 activity, although it affects leukemic expansion more than leukemic engraftment/initiation. It is currently unclear if this PRC2 activity is only oncogenic in myeloid malignancies and tumor suppressive in lymphoid malignancies or whether this is imposed by the collaborating oncogenic insults like MLL-AF9 or NOTCH1.

6.3.1.4 ASXL1

ASXL1 is putative member of the PcG protein family and was recently identified as an important factor involved in regulating PRC2 activity (Abdel-Wahab et al. 2012). Mutations in ASXL1 have been detected in a variety of hematologic malignancies, like CML, MPN, AML, and MDS (Abdel-Wahab et al. 2011; Shih et al. 2012; Cancer Genome Atlas Research Network. 2013), with a worse overall survival in MDS (Bejar et al. 2011) and AML (Metzeler et al. 2011). These mutations were shown to decrease the stability of the protein, as ASXL1 was undetectable in cells with *ASXL1* mutations (Abdel-Wahab et al. 2012). This suggests that ASXL1 has a tumor suppressive function in these myeloid malignancies. Downregulation of ASXL1 led to upregulation of a gene signature that resembled the gene signature observed when MLL-AF9 is expressed in cells (Abdel-Wahab et al. 2012). The upregulated genes included genes in the posterior HOXA cluster (i.e., HOXA9) that are both classical MLL-AF9 and PcG target genes. These data suggested that ASXL1 might modulate the activity of PcG complexes on the *HOXA* locus. Indeed, knockdown of ASXL1 led to a decrease in H3K27me3 levels on the HOXA cluster, which was due to impaired recruitment of the PRC2 complex, and hence loss of EZH2 activity at the HOXA cluster (Abdel-Wahab et al. 2012). ASXL1 was found to directly interact with members of the PRC2 complex suggesting a direct role for ASXL in PRC2 targeting. In an NRAS

(G12D) model of leukemia, it was subsequently shown that loss of ASXL1 not only increased the self-renewal of leukemic cells but also accelerated the onset of leukemia (Abdel-Wahab et al. 2012). This data is consistent with leukemias induced by MLL-AF9/NRAS(G12D), where the HOX-mediated self-renewal component was supplied by the MLL-AF9 fusion (Shi et al. 2013).

ASXL1 has also been shown to form a complex with the deubiquitinase BAP1, which removes the monoubiquitin from histone H2A at Lysine 119 (Scheuermann et al. 2010). This complex (PR-DUB) which was originally identified in *Drosophila melanogaster* binds PcG target genes and is essential for repression of *Hox* genes in *Drosophila* (Scheuermann et al. 2010). This suggested a model where *ASXL1* mutations interfere with both PRC2 and PRC1 function, coordinately contributing to upregulation of the *HOX* cluster. However, both in mouse and human hematopoietic cells, knockout/knockdown of BAP1 did not induce expression of the *HOXA* genes, and loss of ASXL1 did not result in a decrease in H2AK119Ub (Abdel-Wahab et al. 2012; Abdel-Wahab and Dey 2013; Dey et al. 2012). Using a *Bap1*$^{fl/fl}$*;creERT2* conditional knockout model it was shown that loss of *Bap1* induces a MDS/CMML-like disease (Dey et al. 2012). Interestingly, the authors show that apart from influencing PcG-mediated repression, the ASXL1/BAP1 complex also mediates ubiquitination, and thereby the stability of the epigenetic regulators such as OGT (Dey et al. 2012). OGT mediates O-GlcNAcylation of Ser-112 of histone H2B and can be recruited to CpG-rich transcription start sites of active genes via its interaction with TET proteins. It will be interesting to see how abrogation of these distinct functional pathways of ASXL1 contributes to leukemic transformation in ASXL1 mutant cells.

6.3.2 Polycomb Repressive Complex 1

6.3.2.1 PCGF Paralog Family

From the 6 PCGF family members, the best-described role in leukemic transformation is for PCGF4/BMI1. Although PCGF2/MEL18 expression has been shown to decrease upon differentiation of the leukemic cell line HL60, no causal role has been described yet (Jo et al. 2011). The role of BMI1 has been better documented. The first evidence for a possible involvement of BMI1 in the development of hematological malignancies came from murine models in which BMI1 was identified as a cooperating factor with MYC in the induction of B cell lymphomagenesis (van Lohuizen et al. 1991). Next, Sauvageau and colleagues demonstrated that BMI1 not only determines the proliferative capacity of normal stem cells but also of LSCs (Lessard and Sauvageau 2003). In a mouse model in which coexpression of the oncogenes HOXA9 and MEIS1 resulted in a quick onset of myeloid leukemia, no disease was observed in secondary recipients in a *Bmi1*-deficient background (Lessard and Sauvageau 2003). These data indicated that BMI1 is essential for the maintenance of HOXA9-MEIS1 LSCs in vivo. Similar, serial replating of leukemic

colony-forming units after AML-ETO or PLZF-RARα-mediated transformation was severely hampered by deletion of *Bmi1* (Smith et al. 2011). However, lower proliferation and serial replating of leukemic stem and progenitor cells deficient for *Bmi1* were not observed in leukemias with an MLL-AF9 background (Smith et al. 2011; Tan et al. 2011). In that setting, HOXA9 was strongly upregulated by MLL-AF9 and was able to bypass oncogene-induced senescence in the absence of BMI1, as MLL-AF9 transformation was lost in the $Bmi1^{-/-};Hoxa9^{-/-}$ background (Smith et al. 2011).

While BMI1 is required for maintenance and self-renewal of HSCs, no data has been reported indicating that overexpression of BMI1 is sufficient to induce leukemia as a single event. However, a role for BMI1 during myeloid and lymphoid leukemic transformation has been inferred from studies indicating a correlation between high expression of BMI1 and disease progression in various leukemias and MDS/MPD (Chowdhury et al. 2007; Grubach et al. 2008; Mihara et al. 2006; Mohty et al. 2007; Xu et al. 2011; Yong et al. 2011; de Jonge et al. 2011; van Gosliga et al. 2007; Raaphorst et al. 2000; van Kemenade et al. 2001; Dukers et al. 2004). In primary human acute myeloid leukemia patient samples, BMI1 was among the highest upregulated PcG genes in AML $CD34^+$ cells compared to normal BM $CD34^+$ cells (Fig. 6.3) and downregulation of BMI1 impaired long-term expansion and self-renewal properties of LSCs (Rizo et al. 2009). Upon aging, BMI1 expression goes down in lymphoid progenitors, resulting in an upregulation of $p16^{INK4A}$ and $p19^{ARF}$ (Signer et al. 2008). However, reintroduction of Bmi1 was sufficient to render aged lymphoid progenitor cells susceptible for BCR-ABL-induced transformation (Signer et al. 2008). While one paper indicated that a C18Y polymorphism exists in BMI1 that resulted in an increase in proteasome-mediated degradation (Zhang and Sarge 2009), activating mutations in BMI1 have not been described. Although BMI1 function appears to be predominantly regulated at the expression level in tumor cells, posttranslational modifications have also been reported that alter the activity of BMI1. For instance, AKT-induced serine phosphorylation has been shown to inhibit BMI1-mediated HSC self-renewal, INK4A-ARF repression, and its ability to promote tumor growth (Liu et al. 2012). In contrast, in prostate cancer it was demonstrated that AKT-mediated phosphorylation can enhance the oncogenic potential of BMI1, independent of INK4A-ARF repression (Nacerddine et al. 2012). In AML1-ETO-positive leukemias, it was shown that aberrant signaling via mutated cKit can cause loss of Polycomb-mediated repression (Ray et al. 2013). Together, these data indicate that cytokine/growth factor signaling can directly influence PcG proteins, and it will be very interesting to further delineate the role of posttranslational modifications of PcG proteins in the future.

Over the past decades, a concept has emerged in which leukemia is regarded as a multistep process in which a number of (epi)genetic events are required in order to induce overt disease. As discussed above, to date it has not been demonstrated that expression of BMI1 alone is sufficient to induce leukemia. However, various lines of evidence suggest that BMI1 might act as an important collaborating factor in the transformation process. In a tumor model in which the oncogene TLS-ERG was introduced into human hematopoietic progenitors, in a limited number of cases the

transduced cells underwent a stepwise transformation and immortalization in which upregulation of BMI1 was identified as one of the cooperating hits (Warner et al. 2005). BMI1 can cooperate with H-RAS to induce aggressive breast cancer with brain metastases (Datta et al. 2007). Primary human epithelial cells could efficiently be immortalized by co-expressing hTERT and BMI1 (Haga et al. 2007). One report indicated that some of the X-linked SCID patients transplanted with retrovirally transduced $CD34^+$ BM cells in order to re-express the IL2γ receptor developed T cell leukemias. Integration site analysis revealed that in one patient the vectors had integrated near the *BMI1* gene (Hacein-Bey-Abina et al. 2008), leaving open the possibility that an upregulation of BMI1 might have contributed to the development of leukemia in this patient as well. Together with BCR-ABL, BMI1 is able to induce myeloid and lymphoid transformation in vitro and a serially transplantable $CD19^+$ B-lymphoid leukemia in vivo (Rizo et al. 2010; Waldron et al. 2011). This collaboration between BCR-ABL and BMI1 was recently confirmed in a mouse model where B-lymphoid progenitors were transformed to B-ALL blasts upon overexpression of BMI1 (Sengupta et al. 2012). Where BMI1 induced self-renewal, the collaborating BCR-ABL oncogene prevented apoptosis and maintained proliferation (Sengupta et al. 2012).

Our understanding of the mechanisms by which BMI1 exerts its phenotypes is steadily increasing. While gain of BMI1 function might be involved in extending the lifespan of normal and LSCs by bypassing senescence, more direct control over the fate of HSC divisions appears to exist also. Although the molecular mechanisms remain to be elucidated, the symmetry of cell division of HSCs is directed toward a more symmetric mode of cell division upon overexpression of BMI1 (Iwama et al. 2004). While under normal homeostasis HSCs might divide asymmetrically, resulting in one new HSC and one daughter cell that has lost stem cell integrity and will differentiate, high BMI1 levels might dictate a more symmetric distribution of specific proteins, mRNAs, or other metabolites during mitosis whereby stem cell integrity is maintained in both daughter cells. How BMI1 would be involved in such processes remains unclear.

Protection against oxidative stress and apoptosis emerges as an important BMI1-downstream pathway as well, either by reducing p53 levels via BMI1-mediated repression of the INK4A/ARF locus or via modulation of the oxidative stress response in an INK4A/ARF-independent manner. Downmodulation of BMI1 resulted in an accumulation of ROS levels, both in knockout mouse models as well as in human $CD34^+$ cells transduced with lentiviral BMI1 RNAi vectors (Liu et al. 2009; Rizo et al. 2009). In other non-hematopoietic model systems, it was also shown that downmodulation of BMI1 results in p53-mediated apoptosis, whereby ROS levels were increased (Alajez et al. 2009; Chatoo et al. 2009). The induction of ROS in the absence of BMI1 could be counteracted by treatment with antioxidants such as NAC, but appeared to be independent of INK4A/ARF in hematopoietic cells (Liu et al. 2009). However, in *Atm*-deficient astrocytes, oxidative stress resulted in an increase in ROS levels, which inhibited cell growth via a MEK-ERK1-BMI1-$p16^{INK4A}$-dependent pathway (Kim and Wong 2009). In $Bmi1^{-/-}$ mice, the increase in ROS coincided with an increase in DNA damage

and an activation of the DNA damage repair pathways, and treatment with NAC or removal of CHK2 at least partially restored some the phenotypes (Liu et al. 2009). A number of genes that have been described to regulate intracellular redox homeostasis were found to be derepressed in *Bmi1*$^{-/-}$ mice (Liu et al. 2009). In human CD34^{+} cells, downmodulation of BMI1 coincided with decreased expression of FOXO3 (Rizo et al. 2009). Foxo3a$^{-/-}$ HSCs were defective in their competitive repopulation capacity, lost their quiescence, and displayed elevated ROS levels (Miyamoto et al. 2007). Thus, BMI1 might be required to protect hematopoietic stem/progenitor cells from apoptosis or loss of quiescence induced by oxidative stress conditions. In human leukemias, besides facilitating symmetric stem cell divisions, the LSC might utilize enhanced expression of BMI1 as a mode to protect itself from oxidative stress.

6.3.2.2 CBX Paralog Family

From the five CBX family members, CBX2,4,6,7, and 8, only CBX7 and CBX8 have been described to play a role in leukemia or lymphoma. CBX7 is a PcG member that has been shown to extend cellular lifespan in similar fashion as BMI1 (Gil et al. 2004). CBX7 was demonstrated to bypass senescence through repression of the *Ink4a*/*Arf* and the *Cdkn1a* locus (Gil et al. 2004). Enhanced expression of CBX7 was observed in human follicular lymphomas, and T cell lymphomas also appeared when CBX7 was ectopically expressed in murine lymphoid cells (Scott et al. 2007). In these lymphomas a decrease in p16^{INK4A} and p14^{ARF} expression was observed, suggesting that the CBX7-mediated bypass of cellular senescence contributes to the malignant phenotype. However, the long latency and incomplete penetrance of these lymphomas suggested that increased expression of CBX7 is not sufficient to drive lymphomagenesis on its own and that additional collaborating events are necessary (Scott et al. 2007). Overexpression of CBX7 in 5FU-treated BM cells recently confirmed the contribution of CBX7 to T-cell malignancies, although in 30 % of the malignancies also erythroid and immature leukemias were observed (Klauke et al. 2013).

The CBX family member CBX8 has been shown to interact with AF9 and ENL (Hemenway et al. 2001; Monroe et al. 2011; Garcia-Cuellar et al. 2001; Mueller et al. 2007). These proteins are common fusion partners for the *Mixed Lineage Leukemia* (*MLL*) gene in juvenile and adult leukemias. Although CBX family members are usually viewed as transcriptional repressors, in the context of MLL-AF9, MLL-ENL, and potentially also other MLL fusion proteins, CBX8 is actually required to induce gene expression (Tan et al. 2011). CBX8 was shown to be essential for MLL-AF9-induced HoxA9 expression (Tan et al. 2011), which had been demonstrated to be required for MLL-AF9-induced leukemic transformation (Faber et al. 2009; Zeisig et al. 2004). Indeed, deletion of *Cbx8* completely abolished MLL-AF9- and MLL-ENL-induced leukemogenesis (Tan et al. 2011). Depletion of CBX8 did not affect the binding of MLL fusion proteins to their target promoters, but rather affected the binding of RNA polymerase II and subsequent

promoter activation (Tan et al. 2011). As CBX family members were originally discovered to have repressive functions, this furthermore highlights that oncogenic MLL-AF9 and MLL-ENL fusions alter the function of CBX8.

6.3.2.3 RING1 Paralog Family

For the PRC1 complex member RING1, not much is known regarding its role in leukemia. RING1 expression is generally higher in MDS and AML as compared to normal bone marrow cells (Xu et al. 2011), and particularly within the AML $CD34^+$ compartment, RING1A is significantly upregulated compared to normal BM $CD34^+$ cells with no significant differences in RING1B expression (Fig. 6.3) (Bonardi et al. 2013; de Jonge et al. 2011). By means of protein pulldown assays, RING1 was found to interact with the MLL-fusion partners AF9 and ENL (Monroe et al. 2011; Mueller et al. 2007). This suggested that MLL-AF9 and MLL-ENL could potentially bind PRC1 through RING1 as well. However, for AF9 it was shown that AF9 does not directly bind RING1B, but uses CBX8 as an intermediate that binds both (Hemenway et al. 2001). As also ENL has been shown to bind to CBX8 (Mueller et al. 2007), it is therefore conceivable that CBX8 also acts as an intermediate between MLL-ENL and RING1. Very little is currently known about the different or overlapping roles of RING1A and RING1B in leukemogenesis which will need to be further investigated in detail. Intriguingly, *Ring1b* depletion had no effect on MLL-AF9-mediated transformation (Tan et al. 2011). Since depletion of Cbx8 did severely affect MLL-AF9-induced leukemia (Tan et al. 2011), this suggests that MLL-AF9-induced leukemic transformation might be independent from canonical PRC1 signaling.

6.3.2.4 PHC Paralog Family

Expression analysis of the PHC family member PHC1 in BM mononuclear cells from patients with B-ALL indicated a complete loss of PHC1 expression (Tokimasa et al. 2001). Deletion of *Phc1* in mice leads to a complete block in B cell maturation between the pro-B and pre-B cell stages in neonatal splenocytes (Tokimasa et al. 2001), whereas T cell development appeared normal. This is consistent with its constitutive expression during B cell development and suggests that loss of PHC1 is underlying the B cell developmental arrest in ALL. How PHC1 expression is lost during B-ALL development is unclear, as Southern blot analysis has shown that both alleles were present in the B-ALL samples (Tokimasa et al. 2001). In AML $CD34^+$ cells, PHC1 and PHC3 expression was found to be significantly upregulated in the AML $CD34^+$ compartment (Fig. 6.3) (Bonardi et al. 2013; de Jonge et al. 2011).

6.3.2.5 SCM Paralog Family

Not much is known about a role for the SCML family members SCMH1, SCML1, and SCML2 in leukemic transformation. Grubach and colleagues have investigated the expression of various PcG genes in a panel of 126 AML patients (Grubach et al. 2008). SCML2 appeared to be significantly higher expressed in AML compared with normal bone marrow cells, especially in AML patients with an underlying t(8;21) or inv(16) translocation. We investigated gene expression in a panel of AML patients subdivided in $CD34^+$ and $CD34^-$ fractions and observed that SCMH1 was significantly upregulated and SCML1 was significantly downregulated in AML $CD34^+$ cells compared to normal BM $CD34^+$ cells (Bonardi et al. 2013; de Jonge et al. 2011).

6.4 Noncanonical PRC1 Complexes

Apart from the canonical five-subunit PRC1 complex, various other noncanonical PRC1 complexes have been described. One category of noncanonical PRC1 complexes contains RYBP or YAF2 instead of a CBX subunit and are targeted to chromatin in a manner independent of H3K27me3 (Gao et al. 2012; Tavares et al. 2012). In addition, the noncanonical BCOR and E2F6 complexes contain PCGF1/NSPC1 and PCGF6/MBLR, respectively (Gao et al. 2012; Gearhart et al. 2006; Ogawa et al. 2002; Sanchez et al. 2007; Trimarchi et al. 2001; Qin et al. 2012; Trojer et al. 2011). Recently, the E2F6 complex was shown to have H2AK119 ubiquitination activity through its RING1 subunits and to induce a repressive chromatin structure (Trojer et al. 2011; Gao et al. 2012). Whereas the complex is essential for mouse development, recent knockdown studies in human CB $CD34^+$ cells did not show a dramatic phenotype upon PCGF6 shRNA expression and suggests that PCGF6 does not play an important role in normal hematopoiesis (Qin et al. 2012; van den Boom et al. 2013).

Interestingly, recent whole-exome sequencing approaches in AML have identified recurrent mutations in the BCOR complex subunits *BCOR* and *BCORL1* (Tiacci et al. 2012; Grossmann et al. 2011; Li et al. 2011). BCOR and BCORL1 are large nuclear proteins that act as corepressor of BCL6 or other transcriptional regulators. Translocations have also been described like t(X;17)(p11;q12) resulting in the formation of a BCOR-RARα fusion in a patient with acute promyelocytic leukemia (APL) (Yamamoto et al. 2010). Most of the identified alterations are nonsense mutations, out-of-frame insertions/deletions, or splice site mutations that most likely result in truncated proteins that lack the C-terminal nuclear receptor recruitment motif. This would suggest that BCOR/BCORL1 would act as a tumor-suppressor gene that is inactivated by mutations in a subset of AML (Tiacci et al. 2012; Li et al. 2011). On the other hand, it has been observed that BCOR together with its binding partners PCGF1 and RING1A in the noncanonical PRC1 complex are among the most highly upregulated PcG genes in AML $CD34^+$ cells

compared to normal BM $CD34^+$ cells (Fig. 6.3) (de Jonge et al. 2011), suggesting that these increased expression levels might participate in the process of leukemic transformation. In line with this notion, it was recently shown that ectopic expression of KDM2B, which targets the PRC1.1/BCOR complex to chromatin, is sufficient to transform hematopoietic progenitors (He et al. 2011). Reversely, depletion of *Kdm2b* significantly impaired Hoxa9/Meis1-induced leukemic transformation by mediating silencing of $p15^{INK4B}$ expression through active demethylation of H3K36me2, suggesting that KDM2B functions as an oncogene (He et al. 2011). Recently, KDM2B was shown to tether the BCOR complex to non-methylated CpG islands in developmental genes, enforcing gene repression by RING1-dependent H2AK119 ubiquitination (Farcas et al. 2012; He et al. 2013; Wu et al. 2013). Further mechanistic studies are required to gain further insight into the possible mechanisms by which the noncanonical BCOR complex might contribute to leukemia development.

6.5 Summarizing Remarks

Challenges for the future lie in the further unraveling of gene networks that are under the control of PcG proteins and how regulation of these genes affects the fate of normal hematopoietic and LSCs. It is becoming clear that multiple distinct PRC1 complexes can be composed of (most likely) specific functions, and it will be interesting to determine whether differences in complex composition exist between HSCs and progenitors, or between leukemic and normal stem cells, and ultimately how complex composition might relate to specific target gene regulation. Also, PcG proteins can act independently of the canonical PRC1 complexes, but whether and how these noncanonical PRC1 complexes may participate in leukemia development still needs to be unraveled. Posttranslational modifications of PcG proteins are beginning to be identified, although our understanding of how these affect Polycomb signaling are far from complete. Future studies will help to further delineate the role of PcG proteins in the normal hematopoietic system as well as in the process of leukemic transformation.

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